JOURNAL OF VIROLOGY, Nov. 1999, p. 9303–9313 Vol. 73, No. 11
0022-538X/99/$04.00+0

Generation of Adenovirus Vectors Devoid of All Viral Genes by Recombination between Inverted Repeats

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Received 3 June 1999/Accepted 15 August 1999

Direct or inverse repeated sequences are important functional features of prokaryotic and eukaryotic genomes. Considering the unique mechanism, involving single-stranded genomic intermediates, by which adenovirus (Ad) replicates its genome, we investigated whether repetitive homologous sequences inserted into E1-deleted adenoviral vectors would affect replication of viral DNA. In these studies we found that inverted repeats (IRs) inserted into the E1 region could mediate predictable genomic rearrangements, resulting in vector genomes devoid of all viral genes. These genomes (termed ΔAd.IR) contained only the transgene cassette flanked on both sides by precisely duplicated IRs, Ad packaging signals, and Ad inverted terminal repeat sequences. Generation of ΔAd.IR genomes could also be achieved by coinfecting two viruses, each providing one inverse homology element. The formation of ΔAd.IR genomes required Ad DNA replication and appeared to involve recombination between the homologous inverted sequences. The formation of ΔAd.IR genomes did not depend on the sequence within or adjacent to the inverted repeat elements. The small ΔAd.IR vector genomes were efficiently packaged into functional Ad particles. All functions for ΔAd.IR replication and packaging were provided by the full-length genome amplified in the same cell. ΔAd.IR vectors were produced at a yield of ~10^4 particles per cell, which could be separated from virions with full-length genomes based on their lighter buoyant density. ΔAd.IR vectors infected cultured cells with the same efficiency as first-generation vectors; however, transgene expression was only transient due to the instability of deleted genomes within transduced cells. The finding that IRs present within Ad vector genomes can mediate precise genetic rearrangements has important implications for the development of new vectors for gene therapy approaches.

The starting point for the presented study was an observation made with first-generation adenovirus (Ad) vectors that contained fragments of Ad5 DNA, specifically the va_I (23) or the precursor to the terminal protein (pTP) (26) genes, inserted into the E1 region. The presence of these sequences in addition to the corresponding endogenous gene resulted in the appearance of two viral bands with different buoyant density in CsCl gradients after ultracentrifugation of lysates from infected 293 cells. This phenomenon was interesting, considering the unique mechanism by which the adenovirus replicates and the functional potential of repetitive sequences to mediate genetic rearrangements.

The genomes of Ad2 and Ad5 are double-stranded, linear DNA molecules, approximately 35 kb in length with an inverted terminal repeat sequence (ITR) of 102 bp on each end. Numerous studies in cell-free systems and in infected cells have established that Ad DNA replication takes place in two steps (reviewed in references 2 and 37). In the first stage, DNA synthesis is initiated by pTP. pTP binds as a heterodimer with the Ad polymerase (Pol) to specific sites within the ITRs. Ad DNA replication begins at both ends of the linear genome, resulting in a daughter strand that is synthesized in the 5′ to 3′ direction, displacing the parental strand with the same polarity. Three nonexclusive mechanisms are proposed for the second step, the replication of the displaced parental strand. (i) Displaced single strands can form partial duplexes by base pairing of the ITRs on which a second round of DNA synthesis may be initiated (22, 36). (ii) When two oppositely moving displacement forks meet, the two parental strands can no longer be held together and therefore separate, resulting in partially duplex and partially single-stranded molecules; the synthesis is then completed on the displaced parental strand (21). (iii) Displaced strands, with opposite polarity resulting from initiation at two different molecular ends, can renature to form a double-stranded daughter molecule (37). Elongation of DNA synthesis requires only DNA binding protein (DBP) and Pol. With 20 to 30 bp being synthesized per second, Ad elongation is relatively slow compared to that in the eukaryotic replication systems (which synthesize ~500 bp/s). DBP may stabilize the formation of the panhandle structure and the interstrand renaturation process (39).

Repetitive sequences are a common feature of prokaryotic and eukaryotic genomes. Direct repeats (DR) and inverted repeats (IR) are associated with DNA recombination processes (5, 20, 29). Furthermore, it is thought that IR-induced DNA secondary structures cause pausing of replication by DNA polymerases and reverse transcriptases, resulting in genetic alterations (1, 7, 12, 13, 19, 38).

The unique Ad replication strategy, involving single-stranded replication intermediates, prompted us to investigate in detail whether repetitive homologous sequences inserted into the Ad vector genome would affect replication of viral DNA or whether it would induce genomic rearrangements. In these studies, we have found that, as a result of the replication of E1-deleted Ad vectors containing IR flanking a transgene cassette, a small viral genome is efficiently formed and packaged. These genomes were devoid of all Ad genes. Particles containing this small genome could be separated from virions with full-length genomes by ultracentrifugation in CsCl gradients. In addition to having interesting virological aspects, this finding has practical importance for Ad vector development.

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Ads have a number of properties that make them attractive vehicles for gene transfer. These include highly efficient mechanisms of gene transfer to a large variety of cell types in vivo and the easy production of purified virus at high titers. Highly efficient transduction is mediated by the capsid and core proteins involved in cell attachment and by internalization, endosomal lysis, and nuclear import. Most Ad vectors used for in vivo gene transfer are deleted for E1 genes. The major limitation associated with these E1-deleted vectors has been in short-term expression in vivo, due to the development of immune responses to expressed viral proteins which result in toxicity and viral clearance. In order to overcome some of these problems, Ad vectors have been developed from which almost the entire Ad genome has been deleted. These include vectors with "gutless," almost full-length genomes, which have been shown to mediate stable transgene expression in vivo (32), as well as encapsidated Ad minichromosomes with genomes of ~13 kb, which also have successfully been used for gene transfer in vitro and in vivo (8, 17, 18). Both vector systems require helper viruses and several serial passages for production.

As an application of our finding that IRs can mediate predictable genetic rearrangements within Ad genomes, we demonstrate here the efficient and straightforward production of new vectors representing small Ad genomes devoid of all viral genes, which are packaged into functional Ad capsids.

MATERIALS AND METHODS

Production and characterization of viral vectors. Plasmids. Sequences of the metal responsive element (MRE) and HS-4 element were taken from the plasmids pMRENeo (33) (provided by Richard Palmiter, University of Washington) and pJC5-4 (GenBank accession no. U78775) (a gift from G. Felsenfeld, National Institutes of Health). The human α_1-antitrypsin (hAAT) cDNA linked to a bovine growth hormone (bPA) gene polyadenylation signal was derived from pBShAAT (14).

All Ad shuttle vectors were based on pΔE1sp1a (Microbix, Toronto, Canada). The construction of the vectors Ad.hAATa/b, Ad.Ins1/1a/b, Ad.Ins1/3a/b, Ad.Ins2/1a/b, Ad.Ins2/2a/b, and Ad.IR(G/C) is described elsewhere (25, 35). Vectors containing shortened IRs [Ad.IR(1.0) and Ad.IR(0.5)] were generated by the digestion of pIns2/2 (35) with *Afl*II and *Hin*dIII. The isolated *Afl*II fragment containing shortened insulator sequences and the MREhAAT cassette was blunted (T4 polymerase) and ligated to the *Eco*RV site of pΔE1sp1a [pAd.IR(0.5)]. Partial digestion of pIns2/2 with *Hin*dIII revealed the 4.1-kb fragment containing two shortened insulators flanking the MREhAAT cassette. The fragment was ligated to the *Hin*dIII site of pΔE1sp1a[pAd.IR(1.0)].

For the construction of Ad.IR-bGal, a transgene cassette containing the β-galactosidase (β-Gal) cDNA fused to the simian virus 40 (SV40) polyadenylation signal (SV40pA) was generated. The SV40pA was cut out from pREP4 (Invitrogen, Carlsbad, Calif.) by *Xho*I/*Sal*I digestion and was ligated to the *Xho*I site of pBluescript SK(+) (Stratagene, La Jolla, Calif.), resulting in pBS-SV40pA. A 3.7-kb *Bam*HI fragment containing the bGal cDNA was derived from pCMVbGal (24) and was inserted into the corresponding site of pBS-SV40pA, resulting in pbGal-SV40pA. Ligation of a bGal-SV40pA containing a 4.1-kb *Xba*I/*Kpn*I fragment of pbGal-SV40pA to the corresponding sites of pSLJCb (35) resulted in pJC(1)bGal. pJC(2)bGal was created by insertion of an HS-4 containing a *Spe*I/*Nhe*I fragment derived from pSLJCa (35) into the *Spe*I sites of pJC(1)bGal. In the right orientation, the complete cassette holding two inverted HS-4 elements flanking bGal-SV40pA was cut from pJC(2)bGal by *Spe*I/*Nhe*I digestion and was inserted into the *Xba*I site of pAd.RSV (14), generating pAd.IR-bGal.

Ads. First-generation viruses with the different transgene cassettes incorporated into their E1 regions were generated by recombination of the pΔE1sp1a-derived shuttle plasmids and pJM17 or pBHG10 (Microbix) in 293 cells as previously described (27). For each virus, at least 20 plaques were picked, amplified, and analyzed by restriction digest. Plaques from viruses with the correct genome structure were amplified, CsCl banded, and titered (in PFU per milliliter) as previously described (14, 27). All virus preparations tested negative for replication-competent Ad and bacterial endotoxin (28). Virus was stored at −80°C in a solution containing 10 mM Tris-Cl (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol.

To produce purified ΔAd.IR-1, 293 cells were infected with Ad.2/2a at a multiplicity of infection (MOI) of 10 PFU/cell and were harvested 40 h after infection. Cells were lysed in phosphate-buffered saline by four cycles of freezing and thawing. Lysates were centrifuged to remove cell debris and were digested for 30 min at 37°C with 500-U/ml DNase I and 200-μg/ml RNase A in the presence of 10 mM $MgCl_2$. Five milliliters of lysate was layered on a CsCl step gradient (0.5 ml at 1.5 g/cm^3, 2.5 ml at 1.35 g/cm^3, and 4 ml at 1.25 g/cm^3) and ultracentrifuged for 2 h at 35,000 rpm (rotor SW41). CsCl fractions were collected by puncturing the tube and were analyzed for viral DNA (27) or were subjected to ultracentrifugation at 35,000 rpm for 18 h in an equilibrium gradient with 1.32 g of CsCl per cm^3. The band containing the deleted virus ΔAd.IR-1 was clearly separated (0.5-cm distance) from other banded viral particles containing full-length Ad.Ins2/2a genomes. Fractions containing deleted virus particles were dialyzed against a solution containing 10 mM Tris-Cl (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol and were stored at −80°C. The genome titer of ΔAd.IR-1 preparations was determined based on quantitative Southern analysis of viral DNA purified from viral particles in comparison to different concentrations of a 1.7-kb hAAT-bPA fragment of pBShAAT (for ΔAd.IR-1) according to a protocol previously described (27). Titers were routinely obtained in the range of 3×10^{12} to 8×10^{12} genomes per ml. Assuming one genome is packaged per capsid, the genome titer equals the particle titer. The level of contaminating Ad.Ins2/2a in ΔAd.IR1 preparations was less than 0.1% as determined by Southern analysis, which is consistent with results obtained by plaque assay of 293 cells (fewer than five plaques per 10^6 total genomes).

Primers used for sequencing the ΔAd.IR-1 genome, specific to Ad5 nucleotides (nt) 319 to 338 and 3550 to 3531, were AdF, 5′-TTGTGTTACTCATAGCGCGT, and AdR, 5′-TTCTTTCCCACCCTTAAGCC. The nested primers to obtain the complete sequence of the IR elements in ΔAd.IR-1 were 5′ (nt 552) TGACATTGTTGGTCTGGC and 5′ (nt 947) GAAAAGCTCCAAGATCCC.

Electron microscopy. For examination of viral particles in the transmission electron microscopy studies, CsCl-purified virions were fixed with glutaraldehyde and were stained with uranyl acetate as described previously (27).

Cell culture. SKHEP-1 cells (HTB-52; American Type Culture Collection, Rockville, Md.), an endothelial cell line derived from human liver (10), were grown in high-glucose Dulbecco's modified Eagle medium with 10% fetal calf serum.

Analysis of viral DNA. Lysates from 2×10^5 cells that had developed complete cytopathic effect (CPE) after viral infection or viral material banded in CsCl gradients were digested with pronase (1 mg/ml in a solution containing 10 mM Tris-Cl (pH 7.4), 10 mM EDTA (pH 8.0) and 1% sodium dodecyl sulfate) for 2 h. DNA was extracted with phenol-chloroform and was precipitated in ethanol. DNA samples were then subjected to gel electrophoresis or restriction analysis.

Southern blotting. Cultured cells were washed three times with phosphate-buffered saline before harvesting. For analysis, 10 μg of genomic DNA was digested with restriction endonucleases at 37°C overnight and then electrophoresed in a 0.8% agarose gel and transferred to a nylon membrane (Hybond N$^+$; Amersham, Arlington Heights, Ill.). The blots were hybridized in rapid hybridization buffer (Amersham) with [α-^{32}P]dCTP-labeled DNA probes (>10^8 cpm/μg of DNA). The fragment used for labeling was the 1.7-kb hAAT-bPA fragment of pBShAAT.

hAAT ELISA. hAAT concentrations in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (14).

RESULTS

Considering the unique mechanism by which Ad replicates its genome, involving single-stranded genomic intermediates able to form intra- and intermolecular hybrids, we decided to study in more detail whether repeated sequences inserted into the viral genome would affect replication of viral DNA. To this end, a number of first-generation Ad vectors were used that contained single or repeated copies of a 1.2-kb chicken globin HS-4 element (3) inserted into the E1 region together with a reporter gene cassette (Fig. 1A). These vectors were originally designed for an unrelated study that employed the HS-4 element as an insulator to shield a heterologous, inducible promoter from interference by Ad enhancers (35). Control vectors consisted of the promoterless transgene only (Ad.hAATa) or the transgene expression cassette combined with one HS-4 element (Ad.Ins1/1a and Ad.Ins1/3a). Ad.Ins2/2a and Ad.Ins2/2b contained the HS-4 elements as IRs flanking the reporter gene cassette in leftwards or rightwards orientation, respectively. In Ad.Ins2/1a and Ad.Ins2/1b, the transgene cassette was flanked by HS-4 DRs. In a first screening for abnormal vector replication products, viral DNA was isolated together with chromosomal DNA from infected 293 cells after the development of full CPE and was analyzed by gel electrophoresis (Fig. 1B). The full-length (~35-kb) genome comigrated with fragments of genomic DNA. Interestingly, a small (~5.7-kb) band appeared in DNA samples isolated from cells

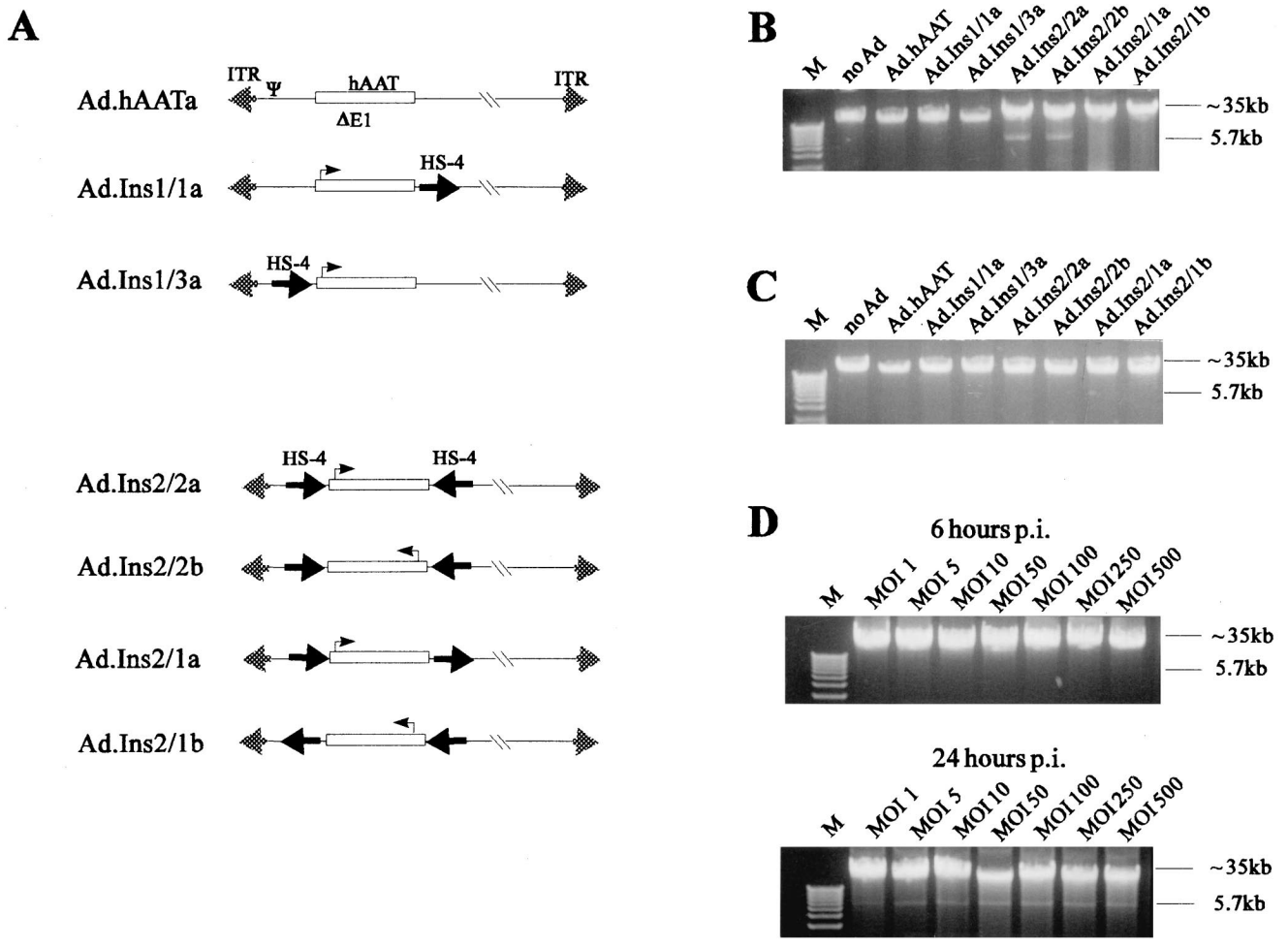

FIG. 1. Formation of genomic derivatives during replication of first-generation vectors carrying two IRs. (A) Structure of first-generation Ad vectors with transgene cassettes inserted into the E1 region. The transgene cassette (hAAT) comprises an inducible MRE promoter (33) linked to hAAT cDNA and a polyadenylation signal derived from the bPA. The IR sequence represents the 1.2-kb fragment of the HS-4 domain derived from the chicken β-globin locus (3). Ad.Ins1/1a and Ad.Ins1/3a contain only one HS-4 element upstream or downstream of the transgene cassette. Ad.Ins2/2a, Ad.Ins2/2b, Ad/Ins2/1a, and Ad.2/1b each contain two HS-4 elements as IRs or DRs, respectively. Ad.hAATa contains only the hAAT cDNA and a polyadenylation signal derived from the bPA. (B) Replication studies. 293 Cells were infected with Ads at an MOI of 10 PFU/cell. Thirty-six hours postinfection (p.i.), total DNA was isolated from infected cells and analyzed (undigested) by electrophoresis in agarose gels stained with ethidium bromide. The sample volume loaded per lane corresponds to the amount of DNA isolated from ~20,000 infected cells. The full-length (35-kb) viral genome comigrates with fragments of cellular DNA. The specific replication derivatives from Ad.Ins2/2a and -b with lengths of 5.7 kb are marked. (C) 293 cells were infected, and DNA was analyzed as in panel B. To inhibit viral replication, hydroxyurea at a final concentration of 10 mM was added to the 293 culture medium 1 h postinfection. (D) 293 cells were infected with Ad.Ins2/2a at different MOIs (PFU per cell). DNA analysis was performed as described in panel B before Ad replication started (6 h postinfection) and after the initiation of replication (24 h postinfection) (34). M, 1-kb DNA ladder (Gibco BRL, Grand Island, N.Y.)

after infection with Ad.Ins2/2a and Ad.Ins2/2b, both of which contained HS-4 elements as IRs. These bands were absent in cells infected with the control vectors or the vectors containing two HS-4 DRs. The 5.7-kb bands were identified as Ad vector derivatives by Southern blotting with a transgene specific probe (data not shown). These derivative genomes are hereafter referred to as ΔAd.IR-1 (for the Ad.Ins2/2a derivative) and ΔAd.IR-2 (for the Ad.Ins2/2b derivative).

Quantitative Southern analysis revealed that $\sim 5 \times 10^4$ of the 5.7-kb ΔAd.IR-1 or ΔAd.IR-2 genomes and $\sim 10^5$ corresponding full-length genomes were produced per cell after infection with the corresponding first-generation vector at an MOI of 10. The appearance of these small vector genomes was linked to adenoviral DNA replication, because it was absent when hydroxyurea, an inhibitor of viral DNA replication, was added to the 293 culture medium after infection (Fig. 1C). The amount of ΔAd.IR-1 genomes produced was analyzed 6 and 24 h after infection of 293 cells with different MOIs of Ad.Ins2/2a (Fig. 1D). ΔAd.IR-1 genomes were absent when cell lysates were analyzed before replication started (6 h postinfection). At 24 h postinfection, the number of ΔAd.IR-1 genomes increased when the viral dose was between MOI 1 and 10 and reached a plateau after infection with higher MOIs (50 to 500).

DNA restriction analysis and sequencing of the 5.7-kb viral genomes revealed the genome structure shown in Fig. 2A. Notably, the 4.0-kb *Not*I and the 1.4-kb *Bam*HI fragments were specific for ΔAd.IR-1 and ΔAd.IR-2 and were absent from the full-length genome and the original shuttle plasmid (Fig. 2B). Both deleted vectors, ΔAd.IR-1 and ΔAd.IR-2, contained the transgene cassette flanked by the inverted HS-4 elements, which are linked on both sides to two identical inverted copies of Ad DNA comprising the Ad ITR and packaging signal. Importantly, these genomes were devoid of all Ad sequences that encode viral proteins. This structure was confirmed by sequencing the *Not*I fragments of both ΔAd.IR genomes with

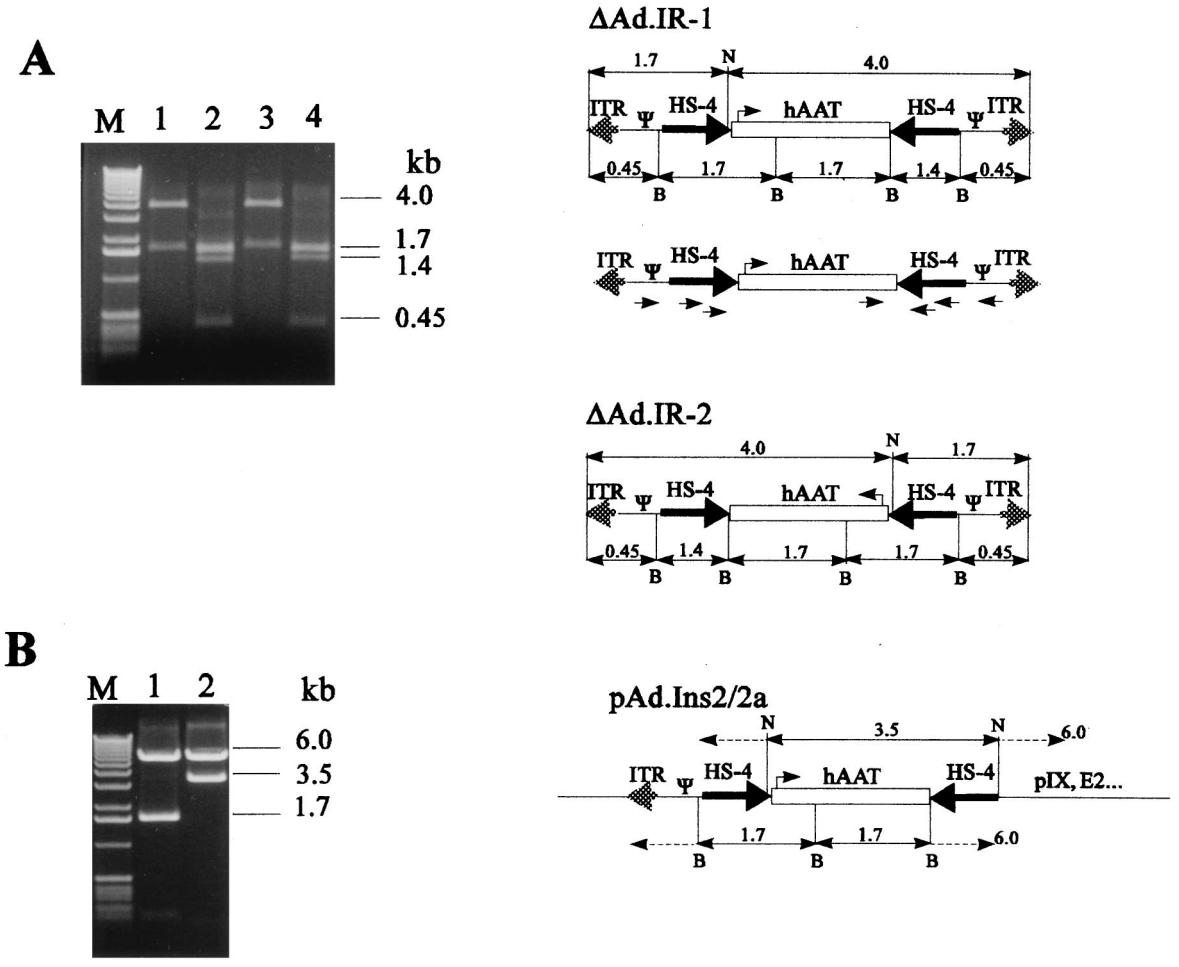

FIG. 2. Structure of ΔAd.IR-1 and ΔAd.IR-2 genomes. (A) ΔAd.IR-1 and Ad.IR-2 genomes were excised from gels after gel electrophoresis, were purified, and were submitted to restriction analysis or sequencing with primers specific to Ad sequences or to the transgene cassette (see Materials and Methods). The results of restriction analysis of ΔAd.IR-1 (lanes 1 and 2) and ΔAd.IR-2 (lanes 3 and 4) genomes after digestion with *Not*I (N) (lanes 1 and 3) or *Bam*HI (B) (lanes 2 and 4) are shown. The structures of ΔAd.IR-1 and ΔAd.IR-2 genomes were deduced from restriction and sequencing data. The localization of sequencing primers specific to nt 319 to 338 within the Ad region is indicated in the lower chart for ΔAd.IR-1. (B) For comparison, the *Bam*HI (lane 1) or *Not*I (lane 2) digest of the corresponding pΔE1sp1a-based shuttle plasmid used for generation of Ad.Ins2/2a is shown. The 3.6-kb *Not*I and 1.7-kb *Bam*HI fragments are specific for the full-length viral Ad.Ins2/2a genome. Due to additional restriction sites within the viral genome, the restriction pattern is only shown for the shuttle plasmid. The 6.0-kb fragment contains the pΔE1sp1a backbone. Ψ, Ad packaging signal; IR, HS-4 IR; hAAT, transgene expression cassette; pIX, gene for Ad pIX protein. All fragment sizes are in kilobases. M, 1-kb DNA ladder (Gibco BRL).

primers specific to the Ad packaging region or primers specific to regions within the transgene cassette (Fig. 2A). The sequencing data demonstrated an accurate mechanism for the duplication of the IRs in conjunction with the Ad packaging signal and ITR.

The appearance of ΔAd.IR genomes with identical, duplicated regions was linked to viral DNA replication and required the presence of IRs. DRs did not mediate this process. Based on these results, we hypothesized that the unique structure of ΔAd.IR could be the result of homologous recombination processes stimulated by the IRs flanking the transgene cassette (Fig. 3). This process could involve the formation of a Holliday structure, which can be resolved by a classical isomerization process (11, 16) or during Ad replication.

If this model is correct, recombination products should appear in cells coinfected with two Ads; one containing a sequence in leftward orientation, and the other containing an identical sequence in rightward orientation with respect to the Ad ITR and packaging signal (Fig. 4A). To test this hypothesis, vectors Ad.Ins1/3a and Ad.Ins1/3b containing one HS-4 element and the hAAT transgene cassette in leftward or rightward orientation, respectively, were added onto 293 cells separately or in combination. Viral DNA was analyzed together with cellular DNA after development of CPE, as described in Fig. 1. As expected, no small vector derivatives were detected in cells infected separately with Ad.Ins1/3a or Ad.Ins1/3b. Importantly, a 4.2-kb deleted vector genome was generated in cells after simultaneous infection with the two vectors (Ad.Ins1/3a plus Ad.Ins1/3b; Fig. 4B). This product can only form when the two double-stranded genomes (Ad.Ins1/3a and Ad.Ins1/3b) recombine via the HS-4-hAAT homology region. The amount of 4.2-kb deleted vector genomes was similar to the amount observed for ΔAd.IR-1/2 (Fig. 1A). A corresponding recombination product appeared in cells coinfected with Ad.Ins1/1 and Ad.Ins1/1b containing the hAAT cassette followed by the HS-4 element. To demonstrate that recombination is not associated with the specific sequence or structure of the HS-4 element and that recombination can be mediated by other sequences, vectors Ad.hAATa and Ad.hAATb were employed. These vectors contained 1.4-kb hAAT cDNA seg-

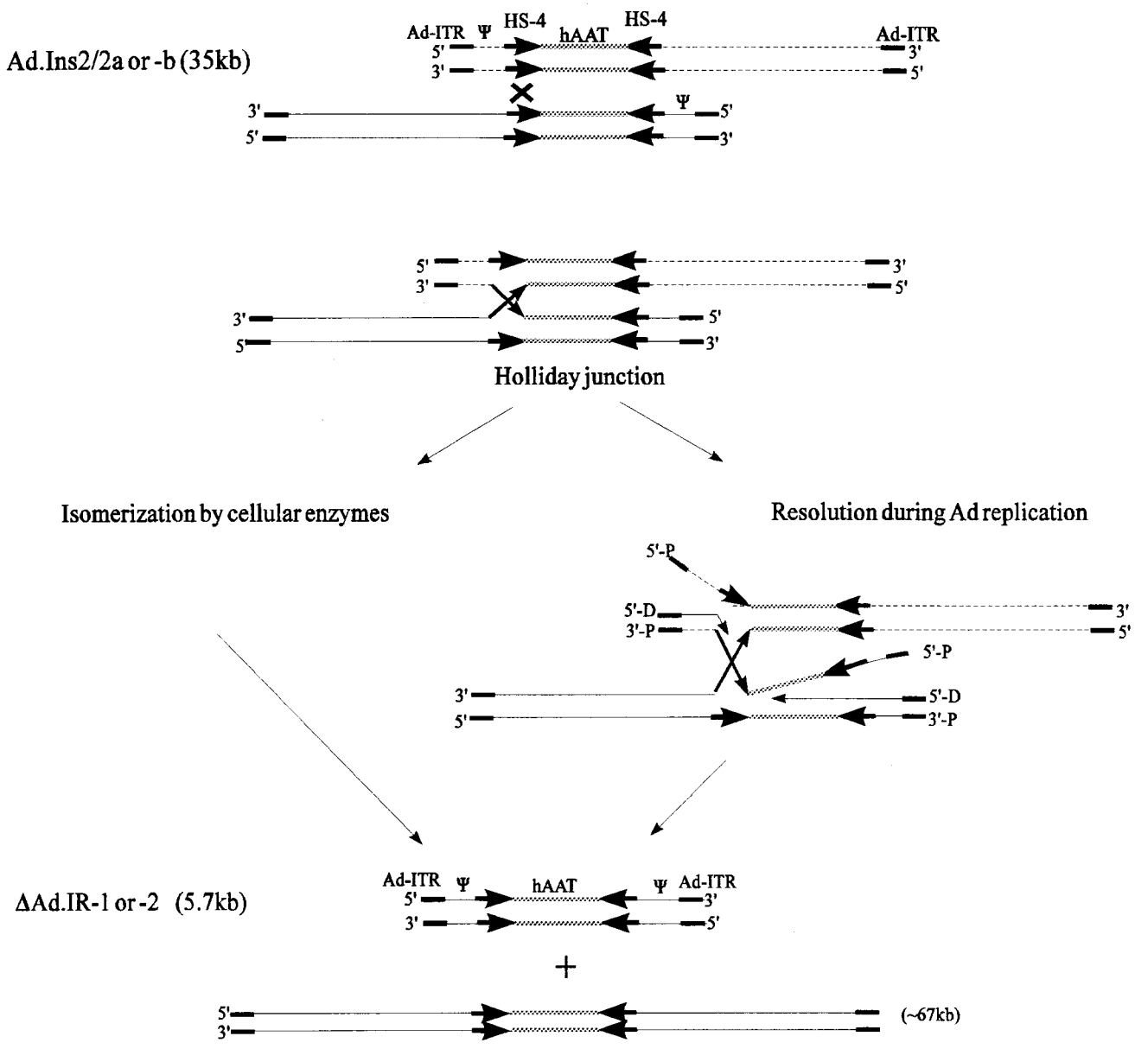

FIG. 3. Hypothetical mechanism for the formation of ΔAd.IR genomes. According to the accepted model for general recombination, the recombination process could begin with the pairing of homologous regions within the HS-4 elements of two double-stranded Ad genomes containing IRs (e.g., Ad.Ins2/2a). Then, cellular recombination enzymes would mediate the exchange of a single strand between the two double-stranded viral genomes, resulting in a Holliday junction. This structure could isomerize by undergoing a series of rotations, as described for the classical Holliday resolution model (11, 16). Alternatively, the Holliday structure could be resolved during Ad replication. The synthesis of a daughter strand (5′-D) is associated with the displacement of one parental strand (5′-P) and could occur along the crossed strands. When two oppositely moving displacement forks meet at the crossover, the two crossed parental strands will no longer be held together and will separate, resulting in partially duplex and partially single-stranded molecules. The synthesis is then completed on the displaced parental strand. A similar dissociation mechanism is described for Ad2 replication (21). One of the recombination products has the structure of ΔAd.IR genomes. Theoretically, a second double-stranded product, with a length of ~67 kb, should also form. We were not able to demonstrate the presence of this product by Southern blotting. This product is apparently not efficiently generated. Its large size would require extremely long periods of time for replication (>40 min). Based on the predicted structure, the 67-kb product would lack packaging signals. Furthermore, the large size of this product would prevent packaging. The figure shows the pairing and the cross-exchange for only one HS-4 pair. With the same likelihood, recombination can occur between the other HS-4 pair, resulting in identical products. To simplify the Holliday resolution model by Ad replication, DNA synthesis initiated from only one genome end is shown.

ments linked to 0.3-kb bPA polyadenylation signals in left- or rightward orientation. In cells coinfected with Ad.hAATa and Ad.hAATb, this 1.7-kb hAAT-bPA region of homology also efficiently mediated the formation of small deleted vectors. The structure of the deleted genomes formed after coinfection with two viruses was confirmed by restriction analysis. Representative data were shown in Fig. 4B.

In conclusion, vectors deleted for all viral genes are efficiently formed by a process that appears to involve homologous recombination between two IRs present in one vector or by recombination between independent vectors, each containing one inverse homology element. Importantly, in contrast to recombination within a single parental vector carrying two IRs, recombination between two coinfected vectors results in the formation of a hybrid ΔAd.IR carrying elements from both parental vectors.

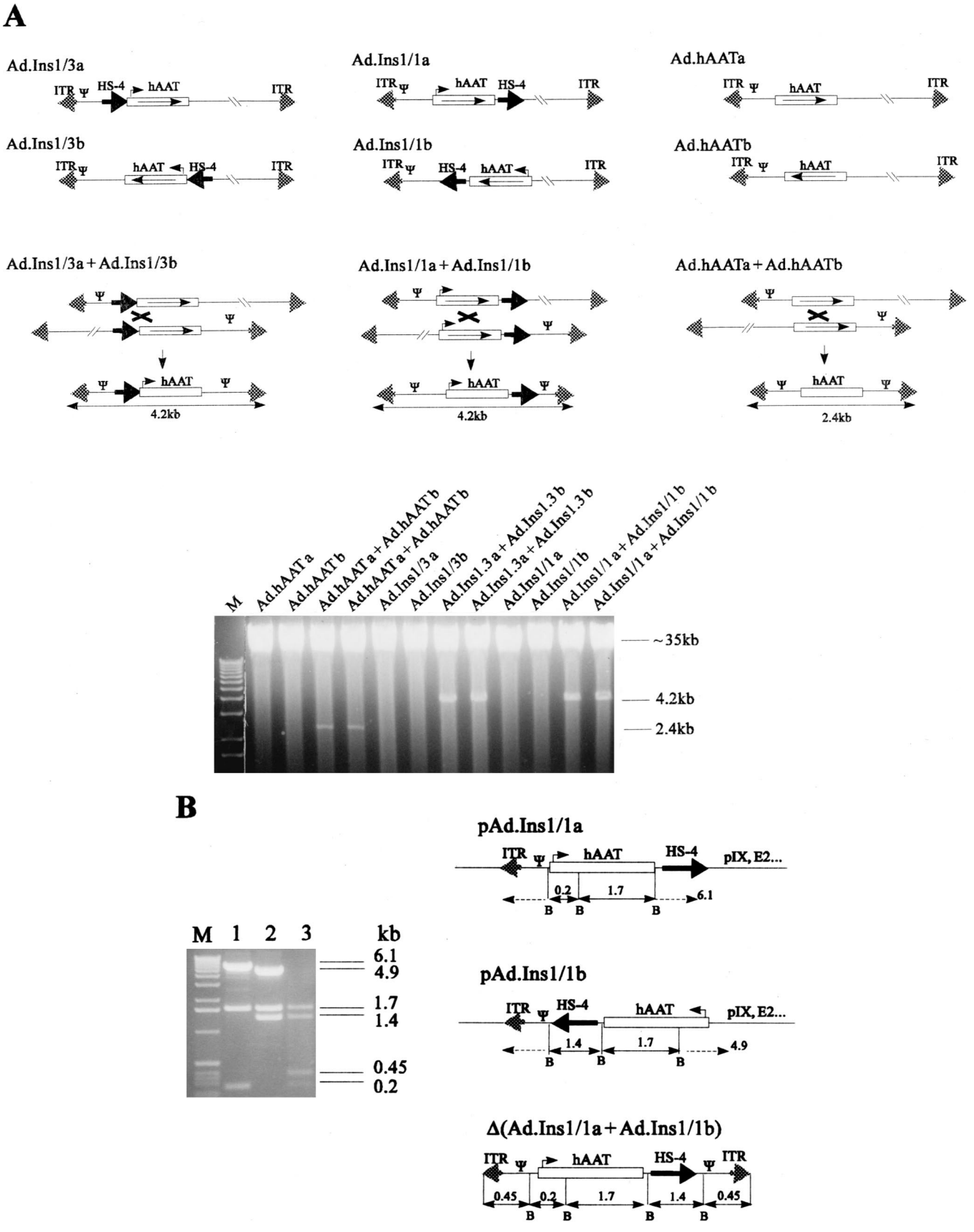

FIG. 4. Formation of genomic derivatives by coinfection of two parental vectors each carrying only one homology element. (A) The structures of Ad.hAATa, Ad.Ins1/1a, and Ad.Ins1/3a are described in Fig. 1A. Ad.hAATb, Ad.Ins1/1b, and Ad.Ins1/3b contain their individual inserts in opposite orientations with respect to their counterparts Ad.hAATa, Ad.Ins1/1a, and Ad.Ins1/3a. Coinfection of Ad.hAATa/b, Ad.Ins1/1a/b, or Ad.Ins1/3a/b allows for intermolecular recombination and

The recombination model for the formation of the described ΔAd.IR genomes relies on an extended homology between the IRs. In order to assess whether the formation of genomic derivatives quantitatively depends on the length of the homologous elements, additional vectors were generated that contained shorter IR elements than Ad.Ins2/2a (Fig. 5). The shorter IRs derived from the HS-4 fragments, with lengths of 1.0 or 0.5 kb, allowed for the generation of the ΔAd.IR genomes at the same rate as the 1.2-kb HS-4 elements used in the experiment described in Fig. 1A. The corresponding replication derivatives had the expected sizes of 3.9 and 4.9 kb, respectively. However, a vector that contained very short IRs consisting of stretches with 20 bp of dC and dG did not yield detectable replication derivatives, indicating that certain lengths of IRs are required for ΔAd.IR formation. Notably, both the 1.0- and 0.5-kb-long IRs were deleted for a GC-rich region located at the terminal ends of the 1.2-kb HS-4 fragments (3) (Fig. 5). Together with the data shown in Fig. 4, this suggests that formation of ΔAd.IR genomes can be mediated by any sequence present as an IR in the E1 region of Ad vectors. To test whether the sequence of the intervening region between the IRs is critical for the formation of genomic derivatives, a vector was produced that had two inverted 1.2-kb HS-4 fragments flanking a 4.1-kb β-Gal gene. During replication of this vector, the expected 8.2-kb genome was formed as efficiently as the genome of Ad.IR-1 or Ad.IR-2. This demonstrates that IRs can be employed for the generation of deleted vectors containing a transgene cassette of choice.

The presence of packaging signals in ΔAd.IR-1 and ΔAd.IR-2 prompted us to test whether these viral genomes were packaged into virions that could be banded in CsCl gradients. 293 cells infected with Ad.Ins2/2a (MOI 10) were lysed 48 h postinfection in order to liberate produced viral particles, and cell lysates were separated by ultracentrifugation in CsCl gradients to band and visualize viral particles. A prominent additional viral band with a buoyant density of ~1.32 g/cm^3 appeared in CsCl step gradients between virions containing full-length (~35-kb) genomes and empty or defective particles (Fig. 6A). Analysis of viral material from purified particles contained in this band demonstrated the packaged 5.7-kb ΔAd.IR-1 genome (Fig. 6B). ΔAd.IR-1 particles could be separated from contaminating particles with packaged full-length genomes (Ad.Ins2/2a) by an additional CsCl equilibrium gradient (Fig. 6B, lane 3). Based on quantitative Southern analysis of DNA isolated from CsCl banded particles, ~10^4 packaged ΔAd.IR-1 genomes and ~5×10^4 packaged full-length genomes were produced per cell (data not shown). Considering the amount of corresponding genomes found at the time of virus harvest inside the cell (Fig. 1B), this indicates that both the 5.7- and the 35-kb genomes were packaged efficiently. Final preparations of ΔAd.IR-1 after two CsCl gradient purifications contained less than 0.1% contaminating first-generation Ad.Ins2/2a, as analyzed by plaque assay (see Materials and Methods). CsCl purification of other ΔAd.IR vectors resulted in similar findings (data not shown).

Electron microscopy of ΔAd.IR-1 particles demonstrated the same icosahedral shape as the first-generation, Ad.Ins2/2a, particles (Fig. 7). Staining with uranyl acetate allows the central viral cores to appear electron dense. While the lumina of particles containing the full-length genomes were homogenously electron dense, virions containing the smaller genomes had only spotted luminal staining, indicating the presence of less packaged DNA in ΔAd.IR-1 particles. We speculate that only one deleted genome is packaged per virion.

As a further test for the intactness of ΔAd.IR-1 particles, we measured the ability to mediate gene transfer into cultured cells based on reporter gene (hAAT) expression (Fig. 8). Confluent SKHEp-1 cells were infected with purified ΔAd.IR-1 and Ad.Ins2/2a particles at an MOI of 2,000 genomes per cell. This cell line does not significantly support the replication of first-generation Ad (30). The level of hAAT expression at day 3 after infection was comparable for both vectors, indicating that in vitro gene transfer was similarly efficient. While transgene expression from the full-length vector was stable during the analyzed time period (7 days), hAAT expression declined gradually for ΔAd.IR-1 starting at day 4 postinfection. Southern analysis of viral DNA isolated from infected cells revealed that the short duration of transgene expression was due to the instability of ΔAd.IR-1 genomes within transduced cells (Fig. 9). The concentration of full-length viral genomes (Ad.Ins2/2a) was comparable in cells harvested at day 1 and day 7 postinfection. In contrast, while the input concentration of ΔAd.IR-1 genomes analyzed at day 1 postinfection was as high as for first-generation vectors, the number of ΔAd.IR-1 vector genomes was barely detectable in transduced cells at day 7 postinfection.

In conclusion, small ΔAd.IR genomes are efficiently formed and packaged during replication of E1-deleted Ad vectors containing two IRs flanking a reporter gene cassette. The mechanism of formation of ΔAd.IR requires viral DNA replication and most likely involves homologous recombination. ΔAd.IR formation can be achieved using IRs of various lengths and origins. The inverted homology elements required for ΔAd.IR generation can also be provided in *trans* by the coinfection of two independent viruses, resulting in a hybrid ΔAd.IR. Particles containing the small genomes devoid of all viral genes could be separated from virions with full-length genomes based on their lighter buoyant density. These particles infected cultured cells with the same efficiency as first-generation vectors; however, deleted genomes were only short-lived within transduced cells. The production of high titers by using two IRs is technically straightforward and does not require helper viruses, because all functions required for the replication of the small genome and for particle formation are provided from the full-length genomes amplified in the same cell.

DISCUSSION

We demonstrated that IRs inserted into first-generation Ad vector genomes mediated precise genomic rearrangement, resulting in vector genomes that were devoid of all viral genes and which were efficiently packaged into functional Ad capsids. This finding has practical implications for Ad vector develop-

production of the indicated products, since the inverse homology elements of a/b vector combinations can pair as proposed for vectors carrying IRs (Fig. 3). 293 cells were infected with the indicated vector combinations at an MOI of 50, and viral DNA was analyzed as described in Fig. 1. Infection with single vector types carrying one homology element did not yield any genomic derivatives. Infection with a/b vector pairs generated the expected vector derivatives of 2.4 kb (Ad.hAATa/b) and 4.2 kb (Ad.Ins1/1a/b and Ad.Ins1/3a/b). (B) The structure of the subgenomic (4.2-kb) Ad genome resulting from coinfection of Ad.Ins1/1a and Ad.Ins1/1b was analyzed by *Bam*HI restriction analysis as described in Fig. 2. For comparison, the *Bam*HI digests of the corresponding pΔE1sp1a-based shuttle plasmids used for the generation of Ad.Ins1/1a (pAd.Ins1/1a, lane 1) and Ad.Ins1/1b (pAd.Ins1/1b, lane 2) are shown. The *Bam*HI fragments specific for the 4.2-kb recombination product [Δ(Ad.Ins1/1a + Ad.Ins1/1b)] (lane 3) represent the double 0.45-kb band and the combination of the 0.2-, 1.4-, and 1.7-kb bands.

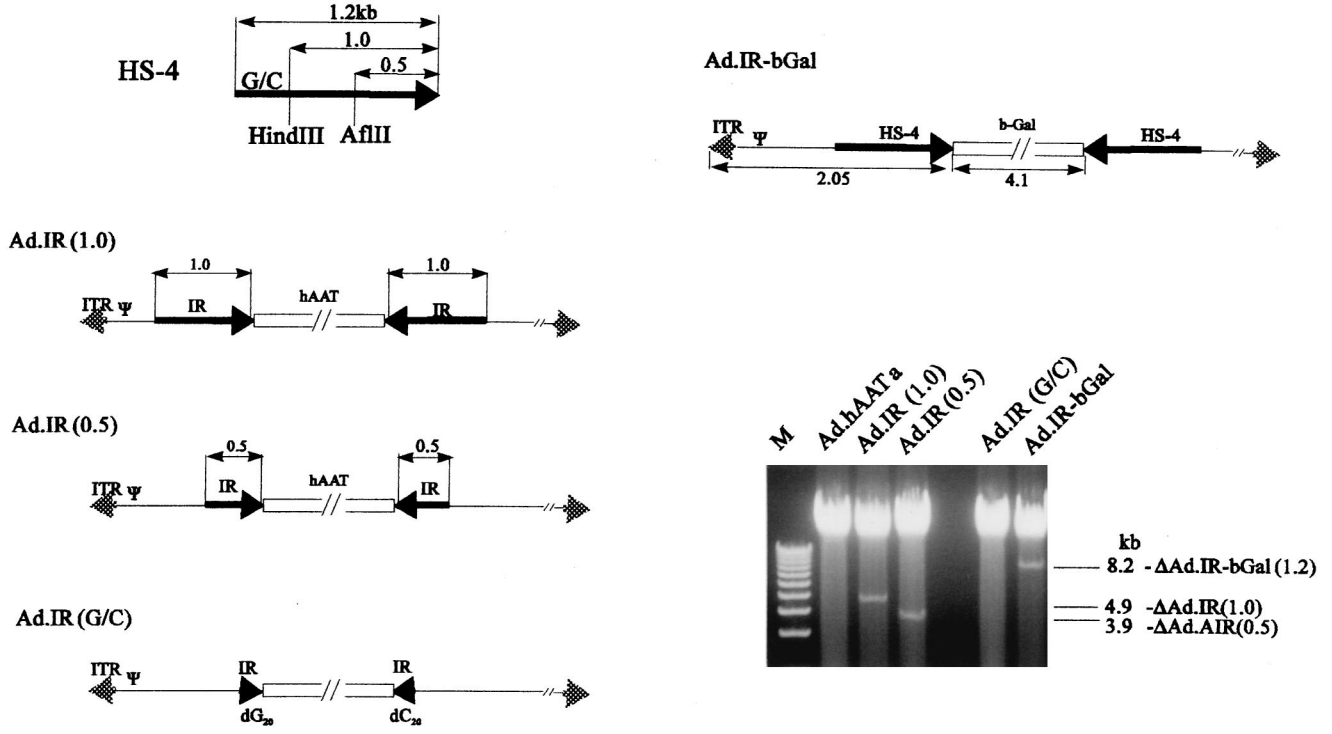

FIG. 5. Effect of shortened IRs on the formation of vector derivatives. 293 cells were infected with Ad vectors, and the viral DNA was analyzed as described in Fig. 1. Ad.IR(1.0) contained HS-4-derived IRs with lengths of 1 kb. Ad.IR(0.5) contained 0.5-kb-long IRs. The restriction sites for *Hin*dIII and *Afl*II present within the 1.2-kb HS-4 element that were used to produce the shorter IRs are indicated. The proximal 200 nt of the HS-4 fragment contain a GC-rich region (chart at the top of the figure). This region and about 150 bp of flanking polylinker are deleted in Ad.IR(1.0) and Ad.IR(0.5). The vector Ad.IR(G/C) contains synthetic 20-mer dG and dC stretches flanking the transgene cassette. The vector derivatives have lengths of 4.9 kb for Ad.IR-1(1.0) and 3.9 kb for Ad.IR(0.5). The structure of the parental vectors is shown on the right. Ad.bGal contained the 1.2-kb HS-4 elements flanking a 4.1-kb *lacZ* cassette inserted into pAd.RSV (14). The corresponding rearranged vector derivative had the expected length of 8.2 kb.

ment and may contribute to a better understanding of Ad replication and the functional importance of IRs.

ΔAd.IR genomes contained only the transgene cassette flanked on both sides by duplicated IRs, Ad packaging signals, and ITRs. This specific structure could be generated precisely and reproducibly by using IRs of different sizes and origins, but not by DRs. These findings implicated the involvement of homologous recombination in the formation process (Fig. 3).

This hypothesis was confirmed by coinfection with two vectors, each containing only one region of homology. The ΔAd.IR genomes detected after coinfection could only have formed by both vectors recombining through oppositely orientated homology elements. This genomic rearrangement process could involve the formation of a Holliday structure whose formation and stabilization could be supported by the Ad DBP, which is known to enhance intermolecular interactions (39). We postulate that the Holliday structure could be resolved by classical isomerization mediated by cellular recombination enzymes, which are highly conserved during evolution (11, 16). Alternatively, the unique mechanism of Ad DNA synthesis may account for the efficient resolution of a Holliday structure, as outlined in Fig. 3. In this context, it would be interesting to test whether similar genomic rearrangements mediated by IRs can be achieved with other DNA viruses (HSV, SV40, or polyomavirus) which use different replication strategies.

At the late stages of viral infection with a relatively low MOI, $\sim 5 \times 10^4$ ΔAd.IR genomes were produced per cell, which is only twofold less than the number of full-length genomes produced per cell. This implies that either the event that forms ΔAd.IR genomes occurs very frequently or that only a small number of rearranged genomes are originally formed and later amplified by the Ad replication machinery. The ΔAd.IR genomes are approximately six times shorter than the full-length genomes and could therefore have a replicative advantage. Previous studies have demonstrated that small Ad vector genomes are replicated by viral proteins expressed from full-length genomes present within the same infected cell (4, 6, 9, 26, 27). This supports the hypothesis that the vector rearrangement is a rare event and that ΔAd.IR genomes are amplified together with full-length genomes in transduced cells. This is further supported by the low frequency of recombination seen between Ad shuttle plasmids used for the generation of recombinant Ads. The critical importance of ΔAd.IR genome replication is also underscored by the observation that the amount of generated ΔAd.IR genomes correlated with the kinetics of Ad replication. The number of ΔAd.IR genomes generated increased with the viral dose between 1 and 10 PFU/cell and reached a plateau when infection MOIs were greater than 10. In this context, it is notable that Ad replication starts only if a certain threshold of early viral protein has accumulated and reaches a plateau that is dictated by limiting viral and cellular factors (37).

Our data does not exclude other mechanisms for the formation of ΔAd.IR genomes. Particularly intriguing is the unique mechanism of Ad replication, involving single-stranded intermediates, that can form intramolecular secondary structures. In this context, stem-loop or cruciform-like structures formed through intrastrand hybridization of IRs may be functionally important in the formation of ΔAd.IR genomes. Elongation by

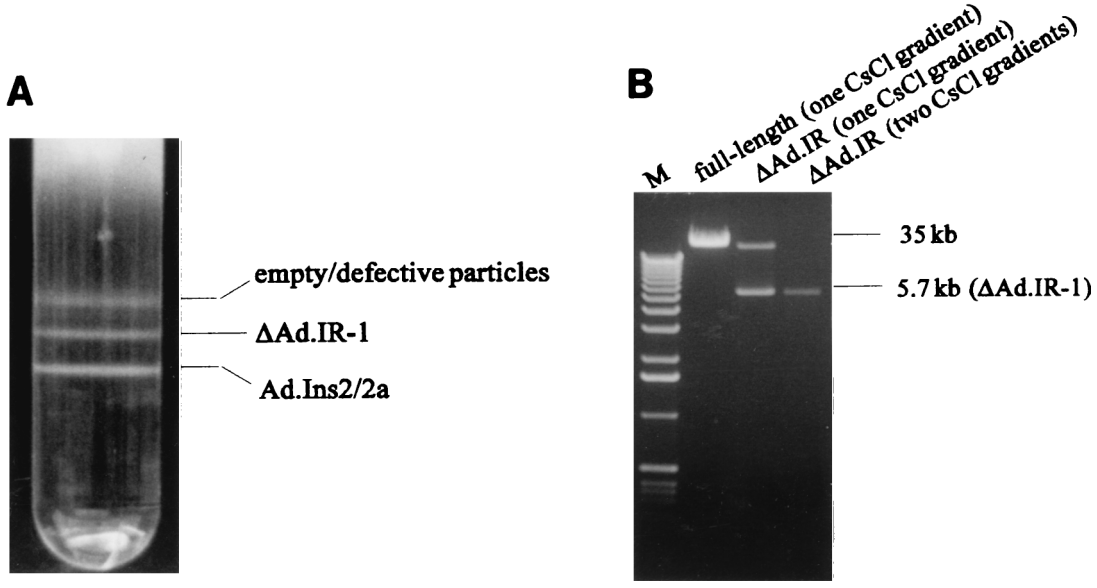

FIG. 6. Isolation of particles containing packaged ΔAd.IR-1 genomes. (A) Ad.Ins2/2a was amplified in 293 cells and banded by ultracentrifugation in a one-step CsCl gradient. The lower band contains full-length genome and Ad.Ins2/2a particles, the middle band contains ΔAd.IR-1 particles, and the top band contains empty and defective viral particles. Viral material from 3×10^8 293 cells collected 36 h after infection with Ad.Ins2/2a at an MOI of 50 PFU/cell is shown. (B) Undigested viral DNA purified from banded viral material after centrifugation in CsCl gradients was analyzed in 0.8% agarose gels. Lane 1, DNA isolated from 10 μl of banded Ad.Ins2/2a (full-length genome, 35 kb); lanes 2 and 3, DNA isolated from 10 μl of the banded ΔAd.IR-1 particles (5.7-kb genome) after a one-step CsCl gradient (lane 2) or after additional purification of ΔAd.IR-1 virions by ultracentrifugation in a CsCl equilibrium gradient (lane 3).

Ad Pol is relatively slow, and it may provide a lag time sufficient to form stem structures within single strands during their displacement. Hypothetically, Ad Pol can pause at the IR-stimulated hairpin structure and switch template strands. Similar mechanisms have been described for other DNA polymerases (1, 13, 19, 38) and for retroviral reverse transcriptases (7, 12, 15). Although the involvement of intramolecular stem-loop or cruciform-like structures formed by IRs present within single-stranded replication intermediates appeared to be an attractive basis for the explanation of the ΔAd.IR structure, the data obtained with coinfected Ad viruses containing only single homology regions contradicted this hypothesis. Nonetheless, local formation of intrastrand secondary structures may initiate or support recombination processes. Clearly, our data demonstrates that Ad replication is required for the high-level production of ΔAd.IR genomes in infected cells, either as the etiological event responsible for the genomic rearrangements or as a supportive mechanism for the amplification of rearranged genomes.

The structure of ΔAd.IR particles revealed by electron microscopy and their buoyant density in CsCl gradients clearly differ from empty particles. Furthermore, we demonstrated that DNase I-treated ΔAd.IR virions efficiently transferred their genomes into cells, as shown by Southern blotting and transgene expression. These facts prove that ΔAd.IR vector genomes, which contain two Ad packaging signals, were packaged into Ad capsids. While the number of deleted ΔAd.IR and corresponding full-length genomes produced per cell differed only by a factor of 2, the ratio of full-length genome particles to ΔAd.IR particles in CsCl gradients was 5:1 to 10:1. This indicates that packaging of the small genomes was 2.5- to 5-fold less efficient than that of full-length genomes. These numbers are in agreement with a study by Parks and Graham (31) in which plasmids carrying Ad genomes of different sizes were used in combination with helper virus to determine the lower packaging limit for Ad vectors. Vectors of fewer than 27 kb were recovered with about half the efficiency of larger vectors. Interestingly, a 15-kb genome was packaged at a higher efficiency than were the 20- to 25-kb-long vectors. However, the work of Parks and Graham demonstrated a clear disadvantage in the amplification of genomes of less than 25 kb during multiple virus passages. Yet, from this experiment, it was not clear whether the smaller vectors were less efficiently replicated or less efficiently packaged. The results of that study are difficult to compare with those of our ΔAd.IR vectors, which start out full length and are deleted in the producer cells (perhaps after a critical event required for packaging has occurred) during one round of large-scale amplification. Packaging of a 9-kb mini-Ad vector generated by Cre-lox recombination (27) or of encapsidated Ad chromosomes (4, 6, 17) has been previously reported.

ΔAd.IR vectors were produced by standard techniques for first-generation adenovirus amplification and purification. All

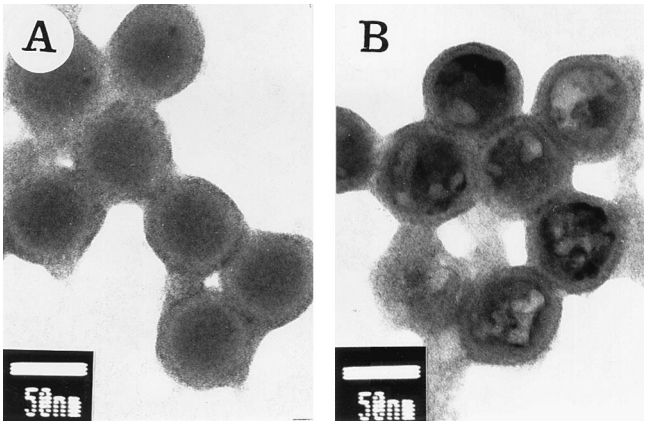

FIG. 7. Electron microscopy of Ad particles. Particles with Ad.Ins2/2a (A) and ΔAd.IR-1 (B) genomes. Virions were purified by two rounds of ultracentrifugation CsCl gradients. Magnification, ×100,000.

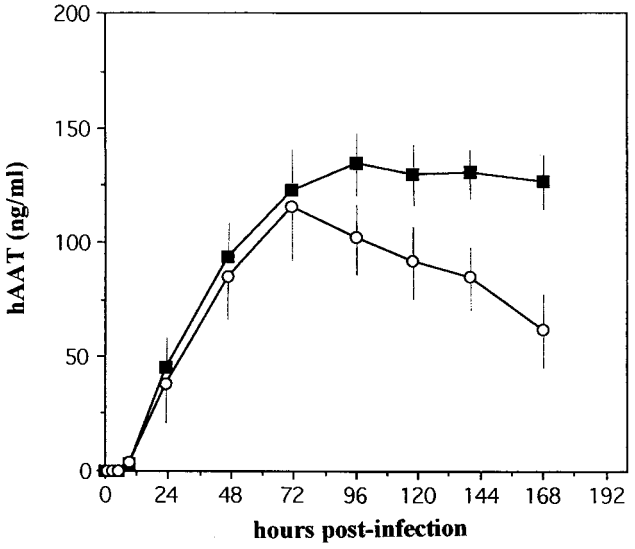

FIG. 8. Expression of hAAT after infection with Ad.Ins2/2a and ΔAd.IR-1. Confluent SKHEP-1 cells (10^6) were infected with purified Ad.Ins2/2a and ΔAd.IR-1 virus at an MOI of 2,000 genomes per cell, and hAAT concentrations were determined in culture supernatants by ELISA. Culture media was supplemented with 150 μM $ZnSO_4$ for induction of hAAT expression and was changed daily. Filled squares, cells infected with Ad.Ins2/2a; empty circles, cells infected with ΔAd.IR-1. Results are based upon three independent experiments.

the functions required for ΔAd.IR genome generation and replication and particle formation are provided from the full-length genomes amplified in the same cell. The efficiency of vector production was 10^4 packaged genomes per cell or $>1 \times 10^{13}$ packaged genomes produced in a large-scale preparation after one round of infection with first-generation vector. Banded particles containing the genomic derivatives were clearly separated in CsCl gradients based on their lighter buoyant density, which allowed for their purification without contamination with first-generation virus containing full-length genomes (Fig. 2B). In this context, the production of high-titer vectors devoid of all viral genes is less labor intensive than helper-dependent production of gutless vectors (32) or packaged adenovirus minichromosomes (17), both of which require multiple passages of virus.

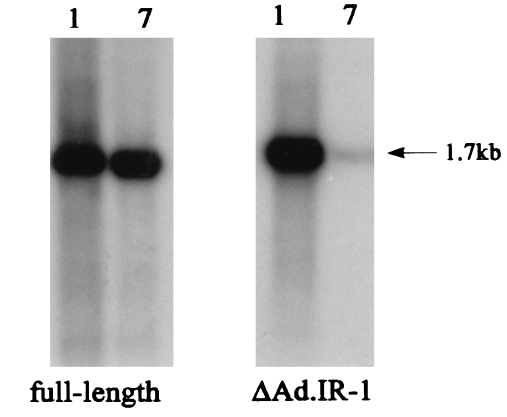

FIG. 9. Analysis of viral DNA in transduced cells. Confluent SKHEP-1 cells were infected with purified Ad.Ins2/2a and ΔAd.IR-1 virus at an MOI of 2,000 genomes per cell. At days 1 and 7 after infection (indicated on top), genomic DNA was extracted. Ten-microgram samples were digested with *Bam*HI and analyzed by Southern blotting with an hAAT-specific probe. The vector-specific fragment is 1.7 kb.

The ΔAd.IR-1 vector infected cells with the same efficiency as the corresponding first-generation vector based on a similar level of reporter gene expression at day 3 postinfection. However, transgene expression declined over time due to the instability of ΔAd.IR-1 genomes in transduced cells. Nonetheless, the high infectivity of ΔAd.IR-1 indicates that the viral structural elements present in ΔAd.IR particles are functionally intact and mediate efficient cell entry, endosomal lysis, and nuclear import. This may allow for the efficient infection of a variety of cell types, including nondividing cells. The potential for highly efficient gene transfer, together with the fact that the ΔAd.IR vector genomes lack viral genes, make ΔAd.IR vectors practically important. For example, a transient transgene expression would be sufficient for a variety of cell biology or cell cycle studies which require efficient gene transfer and the absence of side effects associated with the expression of adenoviral proteins.

On the other hand, approaches aimed toward gene therapy of genetic disorders require stable gene expression. In agreement with the data presented here, we recently demonstrated that a 9-kb mini-Ad genome generated by Cre-lox recombination was packaged into Ad particles that transduced cells efficiently; however, they were short lived and were completely degraded by day 7 after in vitro infection (27). We suggested that the expression of certain viral proteins, including pTP, is required to confer genome stability in transduced cells (26). Furthermore, in a study related to the present paper, we utilized adeno-associated virus elements in combination with the described rearranged vectors to mediate integration as a means of vector stabilization allowing for stable transgene expression.

The strategy of ΔAd.IR generation using coinfection of viruses each containing one inverse homology element can be used to combine elements of choice from two independent viruses. Because the packaging capacity of both parental vectors could be exploited, large inserts could be accommodated by ΔAd.IR vectors. In order to test this, we are currently generating ΔAd.IR vectors containing 12- to 15-kb transgenes. Furthermore, promoter and transgene could be placed into separate vectors so that the transgene would not be expressed during generation and amplification of the parental vectors unless both vectors were coinfected. This strategy may be extremely useful whenever transgene expression is toxic to producer cells.

This study demonstrates proof of the principle that IRs can be used to create predictable genetic rearrangements within the framework of Ad replication. This method allows for the reliable and efficient generation of vectors devoid of all viral genes and has potential application in the development of vectors for gene therapy.

ACKNOWLEDGMENTS

We thank Zong-Yi Li for technical assistance. We are grateful to Dmitry Shayakhmetov and David Russell for critical discussion.

This work was supported by the Cystic Fibrosis Foundation and NIH grants R01 CA80192-01 and R21 DK55590-01. D.S.S. is a recipient of a predoctoral DAAD fellowship. C.A.C. is supported by a grant from the University of Washington (Department of Environmental Pathology and Toxicology).

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